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Somatotropin response *in vitro* to corticosterone and triiodothyronine during chick embryonic development: Involvement of type I and type II glucocorticoid receptors

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Abstract

Corticosterone (CORT) can stimulate growth hormone (GH) secretion on embryonic day (e) 12 in the chicken. However, CORT failed to induce GH secretion on e20 in a single report, suggesting that regulation of GH production changes during embryonic development. Secretion in response to CORT during embryonic development is modulated by the thyroid hormones triiodothyronine (T_3) and thyroxine (T_4). Growth hormone responses on e12 involve both glucocorticoid (GR) and mineralocorticoid receptors (MR); however, involvement of MR has not been evaluated past e12. To further define changes in somatotroph responsiveness to CORT, pituitary cells obtained on e12–e20 were cultured with CORT alone and in combination with T_3 and GH-releasing hormone (GHRH). Growth hormone mRNA levels and protein secretion were quantified by quantitative real-time polymerase chain reaction (qRT-PCR) and radioimmunoassay (RIA), respectively. Corticosterone significantly increased GH mRNA and protein secretion on e12; however, mRNA concentration and protein secretion were unaffected on e20. Contributions of GR and MR in CORT responses were evaluated using GR and MR antagonists. Treatment with a GR-specific antagonist effectively blocked the CORT-induced increase in GH secretion on e12. The same treatment on e20 had no effect on GH secretion. These findings demonstrate that GR is directly involved in glucocorticoid stimulation of GH secretion at the time of somatotroph differentiation but is not regulatory at the end of embryonic development. We conclude that positive somatotroph responses to CORT are lost during chicken embryonic development and that GR is the primary regulator of CORT-induced GH secretion.

Keywords: Growth hormone; Thyroid hormone; Glucocorticoids; Glucocorticoid receptor

1. Introduction

Growth hormone (GH) is produced in the anterior pituitary by cells known as somatotrophs and regulates long bone growth, protein synthesis and muscle accretion, lipolysis, and immune function [1–3]. Defects affecting the actions of GH result in dwarfism and the misutilization of nutrients [3]. In the juvenile chicken, pulsatile GH affects long bone growth, feed efficiency, circulating T_4, and body composition due to a reduction in abdominal fat pad weight and an increase in body mass [4]. However, the mechanism of action initiating GH secretion is still unknown.

Current research in our laboratory centers on the onset of GH secretion, differentiation of somatotrophs, and the extrapituitary signals that regulate this phenomenon using the chicken as a model. The chicken is a unique

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model for studies of pituitary development because there is little maternal endocrine interaction as in mammals, and the egg allows for easy manipulation of the embryo for a multitude of experiments. Furthermore, it is more feasible to obtain the large number of embryonic pituitary cells necessary for cell culture experiments than from common mammalian models.

The adrenal glucocorticoid CORT is an extrapituitary signal that induces final somatotroph differentiation in embryonic chickens, which typically occurs on embryonic day (e) 14 [5,6]. Plasma CORT and adrenocorticotropic hormone (ACTH) dramatically increase between e11 and e17 [7], mimicking the pattern of somatotroph abundance and preceding the increase in plasma GH levels. Administration of CORT on e11 *in ovo* induces somatotroph differentiation and significantly increases GH-secreting cells on e14 and e18, as determined by reverse hemolytic plaque assay (RHPA) [8]. The combination of CORT injection and GHRH treatment significantly increased GH secretion by cultured e12 pituitary cells but not by cultured e17 pituitary cells [9]. Dispersed pituitary cells from e16 and e18 but not e20 embryos acutely treated with CORT show an increase in GH-secreting cells [10]. However, the methods used in these studies were different, and thus the apparent loss of responsiveness to CORT is difficult to interpret. Therefore, the differences reported between studies of somatotroph responsiveness to CORT on e12 and a lack of somatotroph responsiveness to CORT on e20 may be an artifact of the different methodologies and approaches used among studies. Directly quantifying GH mRNA levels and protein secretion by pituitary cells at specific intervals during the last half of embryonic development with 1 assay type under 1 experimental condition is needed to confirm differences in somatotroph responses to CORT and the thyroid hormones during development.

Somatotroph differentiation and GH secretion are also regulated by the thyroid hormones T_4 and T_3, where T_4 must be converted to T_3 before it is efficacious. The hormone T_3 modulates the CORT response in a biphasic manner [11]. At low doses (0.01 nM), T_3 is slightly stimulatory alone and augments the CORT effect on GH secretion by e11 pituitary cells. At higher doses (1 and 10 nM), T_3 antagonizes the CORT response. Therefore, GH secretion induced by CORT is confounded when T_3 is present.

Recent evidence shows that CORT-induced somatotroph differentiation involves both the type I (mineralocorticoid, MR) corticosteroid receptor and the type II (glucocorticoid, GR) corticosteroid receptor [12]. Further, CORT is able to bind to both GR and MR, albeit with different affinities [13–15]. Investigating CORT induction of somatotrophs using specific GR agonists and antagonists may elucidate the reason for the apparent change in responsiveness to CORT during chick embryonic development. Results from qRT-PCR suggest that GR is expressed early in pituitary development and increases, whereas MR expression decreases from e12 to e17 [16]. The observed change in receptor gene expression during embryonic development may regulate somatotroph responses to glucocorticoids. However, GR and MR gene expression has not been fully characterized during pituitary development.

The objectives of this study were to determine, using a unified approach, whether GH responsiveness to CORT is lost during chick embryonic development and to assess the involvement of GR and MR in this response. In this study, a sandwich enzyme-linked immunosorbent assay (ELISA) and an RIA for chicken GH, and qRT-PCR were used to quantify GH protein secretion and GH mRNA in response to CORT, T_3, and GR, and MR specific antagonists during the last half of chick embryonic development, and pituitary GR and MR mRNA levels were quantified from e10 to post-hatch day (d) 7.

2. Materials and methods

2.1. Animal use and cell culture

Cell culture reagents were purchased from Gibco Invitrogen (Grand Island, NY). Hormones and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. All procedures were approved by the Institutional Animal Care and Use Committee. Animals used in this study were Avian × Avian strain or Ross × Ross strain chicken embryos purchased from Allen's Hatchery (Seaford, DE). Eggs were incubated in a humidified incubator (G.Q.F. Manufacturing, Savannah, GA) at 37.5 °C and 60% humidity. Eggs were set in the incubator such that embryos of different embryonic ages were available for dissection on the same day. Pituitaries were removed from embryonic chickens on specific days of development using a dissecting microscope and then pooled and dispersed with trypsin as previously described [17]. Cells (2×10^5) cells suspended in Dulbecco's modified Eagle's medium (DMEM, serum free) were allowed to attach for 1 h in 24-well culture plates and then 0.5 mL of serum-free culture medium (SFM; a 1:1 mixture of phenol red-free Medium 199 and Ham's F12, supplemented with 0.1% BSA, 5 μg/mL bovine insulin, 5 μg/mL human transferrin, and penicillin/streptomycin) was added. The plates were incubated for 24 or 72 h in the

presence of treatments: CORT and T_3 alone and in combination (1×10^{-9} M). Growth hormone-releasing hormone (1×10^{-8} M) was added to the culture well for the final 6 h of incubation to stimulate the release of GH from the cells. After that time, medium was collected from each well, centrifuged for 10 min at $300 \times g$ to remove any loose cells, and then the supernatants were frozen at −20 °C for later ELISA or RIA analysis.

2.2. Sandwich ELISA for chicken GH

The sandwich ELISA for chicken GH was conducted using a monoclonal antibody (mAb), mAb 6F5 [18], and a polyclonal chicken GH antiserum [17]. Briefly, 96-well Nunc-brand Immunosorp plates were precoated with the monoclonal antibody (1 μg/mL in 50 mM Na_2CO_3, pH 9.6) overnight at 4 °C. All incubations were conducted in a 37 °C incubator with several PBS (phosphate buffered saline; 100 mM phosphate, 78 mM NaCl, 72 mM KCl, pH 7.4) washes between incubation periods. Samples and standards diluted in 0.1 M PBS/0.1% bovine serum albumin (BSA) were added and incubated for 2 h in a 37 °C incubator. The plates were blocked (1 M PBS/0.1% BSA/1% normal goat serum [NGS]) for 30 min and then incubated with a polyclonal rabbit anti-chicken GH antiserum (1:1000) for 90 min. Goat anti-rabbit IgG biotinylated antibody (1:200) was added for 30 min. The plates were incubated with a commercially available goat anti-rabbit avidin–biotin horseradish peroxidase (1:500) for 30 min. After that time, tetra-methyl-benzidine (TMB) was added as the assay substrate for 3 min, and the reaction in all wells was stopped with the addition of 1 M H_2SO_4. The plates were read immediately using a Wallac (PerkinElmer) plate reader with the absorbance set at 450 nm. The secondary antibody, avidin–biotin horseradish peroxidase, and TMB were obtained from commercial kits (Vector Laboratories, Burlingame, CA). The average GH concentration for each treatment was determined from triplicate ELISA wells performed on triplicate cell culture wells. The specificity of both the monoclonal and polyclonal antibodies was validated previously [17,18]. Further validation of the assay included estimates of sensitivity and repeatability, and demonstration of parallelism. Addition of BSA to samples and standards produced parallel standard curves, regardless of the medium in which the samples were suspended. The interassay coefficient of variation (CV) estimated for this assay was 34.2%, whereas the intra-assay CV was 31.5%. With an assay variability of greater than 30%, only 2-fold changes or higher in GH concentration could safely be considered significantly different.

2.3. RIA for chicken GH

An RIA adapted from one previously reported [19] was conducted to determine GH secretion into the cell culture medium. Recombinant chicken GH (cGH) was iodinated with $Na^{125}I$ using a solution of chloramine T. The percentage incorporation was calculated before continuing purification. The labeled GH was purified on a Sephadex G-25 column (PD-10, Pharmacia) and collected for γ counting. A volume of 200 μL of GH standard or unknown test sample was diluted 1:2.5 in assay buffer. An additional 200 μL of diluted antiserum was added. Nonspecific binding and total binding tubes were prepared as well. A volume of 100 μL of 1× radioiodinated cGH (30,000 cpm) was added to all tubes. The tubes were incubated at 4 °C for 48 h. Next, 200 μL of anti-rabbit IgG diluted in PBS plus ethylenediaminetetraacetic acid (EDTA) was added to all tubes and then incubated for an additional 48 h at 4 °C. On the final day, PBS was added to all tubes except the total count tubes, and the tubes were centrifuged for 30 min at $1900 \times g$ at 4 °C. The supernatant was poured off, and the precipitate was counted in a γ counter.

2.4. Quantification of mRNA

Four replicate experiments were conducted as before, where cultured pituitary cells (1×10^6 cells per well) were treated with CORT for a total of 24 h. At that time point, the cells were harvested, snap frozen, and RNA was extracted using the Qiagen RNeasy Mini Kit (Invitrogen, Carlsbad, CA). In addition, whole pituitary glands from e10 to d7 chicks were dissected, pooled, and snap frozen, and RNA was extracted. Total RNA was quantified using both the optical density absorption ratio OD_{260} nm/OD_{280} nm (>1.9) and the RiboGreen Quantitation Kit (Invitrogen). The total RNA was reverse transcribed by Superscript III into cDNA and diluted 1:1.5. A volume of 2 μL of the diluted cDNA sample was used for analysis. Three-step qRT-PCR was used to quantify mRNA levels using a Bio-Rad iCycler and Sybergreen. The final concentration of the reaction mixture was 0.1% Triton X-100, 10 mM Tris–HCl, 50 mM KCl, 1.9 mM $MgCl_2$, 2 U Taq, 10 mM each dNTP, 1 μM each primer, 20 nM fluorescein, and SYBERgreen II. The cycling parameters were 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. All primers used to quantify mRNA are listed in Table 1. Relative mRNA levels for each sample were calculated using the relative Ct method ($\text{level} = 2^{(\text{Ct of the no. RT} - \text{control} - \text{Ct of the sample})}$), as previously described [20]. Growth hormone expression in cultured cells was normalized to the housekeeping gene

Table 1
The primers used for qRT-PCR are listed.

Gene	Forward primer	Reverse primer
GH	CACCTCAGACAGAGTGTTTGAGAAA	CAGGTGGATGTCGAACTTATCGT
GR	TCGTGAAAAGAGAAGGGAACTCA	AAAAACGTCTGGAAGCAAAAGC
MR	TTGCTCCAGACCTGATTTTTGAT	TGGCACAGTTCAAACATTGCA
GAPDH	AGCCATTCCTCCACCTTTGAT	AGTCCACAACACGGTTGCTGTAT
PGK1	CTACATGCTGTGCGAAGTGGAA	GCCAGGAAGAACCTTACCCTCTAG

glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Mineralocorticoid receptor and GR expression in intact pituitaries was normalized to phosphoglycerate kinase (PGK1).

2.5. Statistical analysis

Each experiment was replicated 4–6 times. Statistically significant differences among treatments were determined using a mixed-model analysis of variance (ANOVA) and an *a priori* test of least-significant differences (LSD) in the SAS statistical program (SAS Inc., Cary, NC). Values were considered significant at $P \leq 0.05$. In figures, different letters denote significance at $P \leq 0.05$. Differences among treatment groups of the RIA data were determined using preplanned contrasts. Data from the ELISA were transformed into a log scale to correct for variance and normality prior to statistical analysis and then back-transformed into the geometric mean for graphical presentation. Values reported for ELISA data are the geometric mean and 1 standard error above and below the mean. A regression line was fitted to the basal level of GH secretion across the 5 ages. A second regression line was fitted to the CORT-induced level of GH secretion across the 5 ages, and the point at which the 2 lines were no longer different was designated as the "nonresponsive point." Values from the qRT-PCR are presented as mean fold change over basal levels for GH or relative to expression on e14 for the MR and GR data, when levels of both were greatest. The $\log_2$ transformation of fold change over basal treatment or age-normalized Ct values for each sample was used for statistical analysis in SAS using the same program as previously stated.

3. Results

3.1. Experiment I: basal pituitary GH secretion during the last half of chicken embryonic development

Culture medium from primary cell cultures of pituitary cells obtained from e12, e14, e16, e18, and e20 embryos was collected, centrifuged, diluted in PBS (e14 samples were diluted 1:3, e16 samples were diluted 1:10, e18 samples were diluted 1:25, and e20 samples were diluted 1:50) and frozen. Results of ELISA analysis for GH protein revealed a significant 6-fold increase in secretion between the ages of e14 (0.334 ± 0.067 μg/mL) and e16 (2.220 ± 0.342 μg/mL) and a 2-fold increase between e18 (2.93 ± 0.48 μg/mL) and e20 (7.00 ± 1.15 μg/mL) (Fig. 1A). The interassay CV for all ELISAs was determined to be 34.2%, and the intra-assay CV was 31.5%.

3.2. Experiment II: GH secretion and mRNA expression in response to GHRH, CORT, and T_3, during the last half of chick embryonic development

The objective of the second experiment was to characterize the response of somatotrophs to CORT, GHRH, and T_3 across developmental ages during the last half of development using 1 culture system and 1 assay system, a sandwich ELISA. The dispersed cultured pituitary cells from e12, e14, e16, e18, and e20 embryos were treated with CORT (0.5 nM) and T_3 (0.5 nM) alone and in combination for 24 h. Six hours before the termination of the experiment, designated wells were treated with GHRH (10 nM) to stimulate the release of GH into the cell culture medium. The duration of treatment and concentration of GHRH were chosen because maximum GH secretion was observed between 4 and 8 h after treatment with GHRH [17]. Growth hormone-releasing hormone alone did not elicit an increase in GH secretion on any age (Fig. 1).

Treatment with CORT alone had no effect on basal GH secretion by cultured cells from all ages studied (Fig. 1). However, treatment with CORT followed by GHRH significantly increased GH secretion by e14, e16, and e18 cultured cells, when compared to GHRH alone. An increase was observed with the same treatment on e12-cultured cells; however, this was not a significant effect. Corticosterone alone and in combination with GHRH had no effect on GH secretion by e20-cultured cells. The transitional age from CORT-responsive to

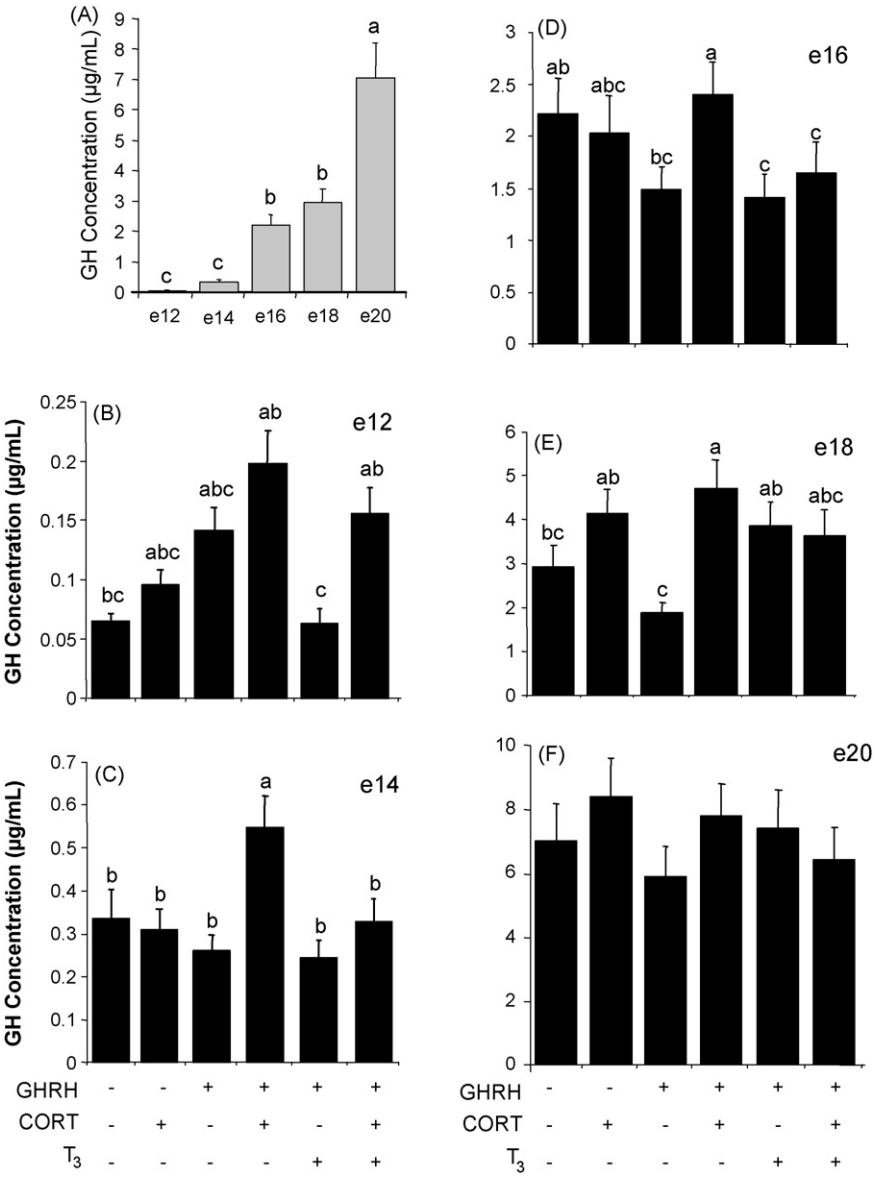

Fig. 1. (A) Basal growth hormone (GH) protein concentration in the cell culture medium as determined by a sandwich enzyme-linked immunosorbent assay (ELISA). Embryonic chicken pituitary cells were cultured for 72 h, and the medium was collected for ELISA analysis. Values presented are the geometric mean and 1 standard error above of 6 replicate experiments. Significant differences were determined using the test of least-significant differences (LSD) of the mean. Different letters above ages represent a significant difference at $P \leq 0.05$. (B–F) Growth hormone protein concentration in cell culture medium in response to treatment with corticosterone (CORT), growth hormone-releasing hormone (GHRH), and T_3 as determined by a sandwich ELISA. Embryonic chicken pituitary cells were cultured for 72 h in the presence of CORT or T_3 alone or in combination. GHRH was added to appropriate wells for the final 6 h of culture to release stored GH, and then the medium was collected for ELISA analysis. Values presented are the geometric mean and 1 standard error above of 6 replicate experiments. Significant differences were determined using the test of LSD of the mean. Different letters denote significant differences at $P \leq 0.05$ within one age group.

CORT-nonresponsive was found to be e16.4 when fitting a regression line to the entire data set.

The response to T_3 changed during development. Growth hormone secretion increased on e18 when T3 was administered at 0.5 nM, but this effect was not observed with any other age (Fig. 1). No effect of T_3 was found in the absence or presence of CORT on e12. T_3 significantly attenuated the CORT response on e14 and e16, but not at any other age. These results indicate that responsiveness to T_3 was lost by e20.

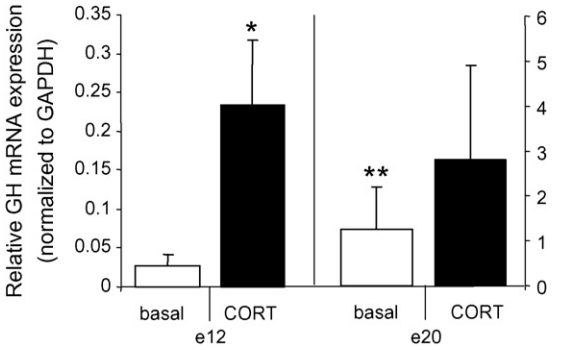

Fig. 2. Relative GH mRNA expression levels normalized to glyceraldehyde 3-phosphate dehydrogenase and represented as mean fold change over e20 basal, as determined by quantitative real-time polymerase chain reaction. Pituitaries dissected from e12 and e20 embryos were dispersed and cultured for 24 h in the presence of corticosterone (CORT) ($n = 4$). Significant differences were determined using the test of least-significant differences of the mean (LSD). *Denotes a significant difference between basal and CORT on e12 at $P \leq 0.05$. **Denotes a significant difference between basal e12 and basal e20 at $P \leq 0.05$.

To determine if the same effect of CORT is seen at the transcription level, GH gene expression was measured by 3-step qRT-PCR and normalized to GAPDH. Corticosterone treatment significantly increased GH mRNA on e12 ($P < 0.01$), but had no significant effect on e20 (Fig. 2).

3.3. Experiment III: determination of GR involvement in the response of cultured pituitary cells to CORT

The specific aim of experiment III was to determine which receptor (GR or MR) is used by CORT to induce GH secretion. To examine receptor gene expression during embryonic development and post-hatch week 1, pituitaries were dissected from e10 to d7 and RNA was extracted. Glucocorticoid receptor and MR mRNA expression in the pituitary gland were quantified by qRT-PCR and normalized to PGK1 (Fig. 3). The pattern of expression of both receptors was similar. Receptor mRNA concentrations peaked on e14, significantly decreased on e16, and maintained a low level of expression through d7.

Since expression levels were similar for both receptors, the next experiment used chemical antagonists of both MR and GR to ascertain involvement of each receptor in CORT induction of GH during chick embryonic development. The objective of this experiment was to determine if both MR and GR are required for CORT responsiveness on e12 and to determine if receptor involvement is a factor in the loss of responsiveness to CORT of pituitary cells late in embryonic development (eg, e20 cells). Since GR and MR both bind CORT and its closely related chemical homologues at different affinities, the chemical antagonists were used at or slightly above their respective effective concentration, as demonstrated in relevant literature [13–15] and a dose response curve from preliminary experiments (data not shown). By using these agents at the concentration of their K_d, it can be assumed that the agonists and antagonists are only activating the receptor toward which they have the most specificity. For this experiment, we used spironolactone (SPIRO) for the MR-specific antagonist and ZK98299 (ZK98) for the GR-specific antagonist. Almost all synthetic and natural glucocorticoids and mineralocorticoids are able to activate both GR and MR when administered at appropriate doses [21,22]. For this reason, we used agents that had at least a 10-fold lower affinity for the opposing receptor. The agent RU486, also known as mifepristone, was not used as a GR-specific antagonist because it has been demonstrated that it is a weak GR agonist and is able to bind to MR [23]. Radioimmunoassay was used instead of ELISA because it is a more sensitive and repeatable assay. The lowest detectable concentration of GH was 0.1 ng/mL as opposed to about 30 ng/mL in the ELISA. The intra-assay and interassay CV were 4.36% and 33.2%, respectively.

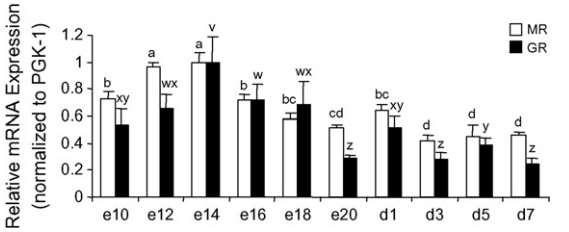

Fig. 3. Relative MR and GR mRNA expression normalized to PGK-1 and shown relative to e14 in the pituitary during chicken embryonic and neonatal development. Pituitaries were dissected from e10 to d7 chickens, and RNA was extracted for mRNA quantitation. Significant differences were determined using the test of least significant differences of the mean (LSD). Values are the mean ± S.E.M. and normalized to e14 levels.

Dispersed chick embryonic pituitary cells (e12 and e20) were cultured for 24 h total and pretreated with the antagonists (SPIRO at 5 nM and ZK98 at 10 nM) for 3 h before the addition of CORT (1 nM), such that the cells were exposed to CORT for 21 h. Growth hormone-releasing hormone was added during the final 6 h of total culture time ($n = 4$). Cultured cells from e12 embryos were responsive to CORT treatment, as seen in the significant 3-fold increase in GH secretion (4.2 ± 1.1 ng/mL) over basal levels (1.2 ± 0.20 ng/mL) (Fig. 4). The 2 antagonists ZK98 and SPIRO, alone and in combination,

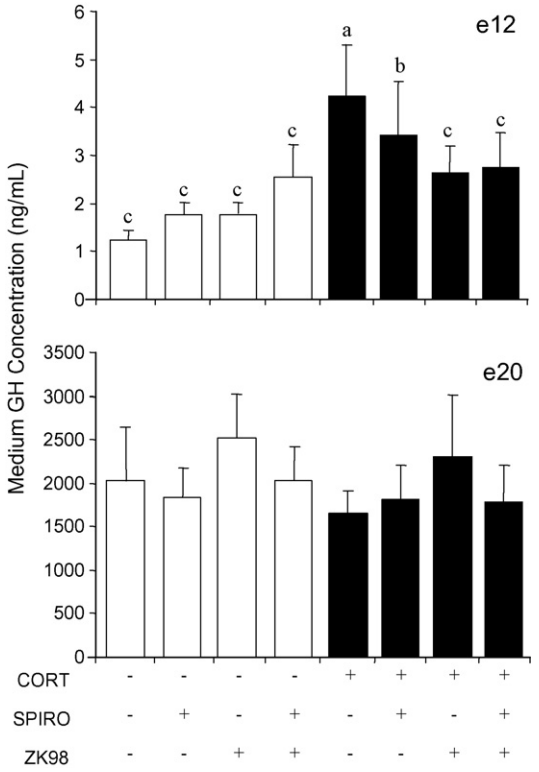

Fig. 4. Growth hormone protein concentration in the cell culture medium in response to the receptor-specific antagonists spironolactone (SPIRO; MR) and ZK98299 (ZK98; GR), as determined by radioimmunoassay. Pituitaries dissected from e12 and e20 embryos were dispersed and cultured for 24 h in the presence of CORT, SPIRO, and ZK98, as indicated. Values are the mean ± S.E.M. of 4 replicate experiments. Significant differences were determined using the test of least-significant differences of the mean (LSD). Different letters above the value represent a significant difference at $P \leq 0.05$.

did not affect GH secretion. Pretreatment with SPIRO followed by treatment with CORT significantly reduced GH secretion into the culture medium, but not as low as basal levels. Pretreatment with ZK98 followed by CORT treatment significantly decreased GH secretion to basal levels. Simultaneous treatment with both receptor antagonists followed by treatment with CORT also significantly decreased GH secretion and was no more effective than pretreatment with ZK98 alone. Repeating this experiment with cultured pituitary cells obtained from e20 chick embryos produced no significant effects (Fig. 4), confirming that GH secretion by e20 pituitary cells was not responsive to CORT.

4. Discussion

Corticosterone significantly increased GH secretion by cultured pituitary cells obtained from e12 through e18 chick embryos, but the effect was lost by e20. This effect was seen only in the presence of GHRH and was modulated by T_3. Corticosterone significantly increased GH mRNA on e12, although this response was not seen on e20. Concentrations of GR and MR mRNA peaked at e14 and slowly decreased during embryonic development. The CORT-induced increase in GH secretion on e12 was blocked by inclusion of ZK98, a GR-specific antagonist, and partially blocked by SPIRO, an MR-specific antagonist. On e20, CORT had no effect on GH secretion, and inclusion of antagonists with CORT had no effect. These data led us to conclude that embryonic somatotrophs are responsive to glucocorticoids, predominantly via GR, until immediately prior to hatching, and this stimulatory effect of CORT is modulated by GHRH and T_3.

The present results demonstrate that CORT induces GH secretion in cultured pituitary cells obtained from e12 to e18 and that this effect is lost by e20. Previous work has demonstrated that CORT can increase the number of GH-producing cells among cultured e12 pituitary cells using immunocytochemistry (ICC) and reverse hemolytic plaque assay (RHPA) [6,9,24], but later ages were not examined in the same study. However, it was previously reported that injection of dexamethasone (DEX), a synthetic glucocorticoid, to e18 chickens had no effect on plasma GH levels 4 and 24 h later [25]. Extensive work has been conducted in rat somatotroph cell lines (GC and GH3 cells) demonstrating that glucocorticoids significantly increase GH mRNA as well as the rate of transcription [26]. Similar experiments demonstrated that DEX is capable of inducing GH secretion in cultured pituitary cells from embryonic rats, although it has not been shown that later embryonic ages are unresponsive to glucocorticoids [27]. Similarly, treatment of pituitary cells of the rainbow trout with DEX showed an increase in GH [28]. In the present study, CORT responsiveness of cultured pituitary cells coincides with the natural increase in circulating levels of glucocorticoids released from the adrenal gland [7]. In embryonic chickens, serum CORT is steady from e10 to e14 and then increases from e14 to e16. Serum CORT again remains constant from e16 to e18, with a final surge from e18 to e20, coinciding with preparation for hatching [7]. In this study and a previous report [10], GH secretion was nonresponsive to CORT on e20, after this endogenous increase in circulating CORT. The concentration of CORT that was chosen for this study is comparable to physiological levels and not pharmacological levels, thus, in theory, we are not introducing an artificial effect of glucocorticoids. Although the pituitary cells obtained from embryos later in development

were unresponsive to CORT in a previous report [10], it was possible that the unresponsiveness was due to an assay artifact. Immunocytochemistry, RHPA, ELISA, and RIA are inherently different in what they measure. Immunocytochemistry estimates GH-containing cells, whereas RHPA demonstrates GH-secreting cells. Neither of these assays quantifies the amount of GH protein secreted, which can be done with an ELISA or RIA. Moreover, the previous findings were determined under varying experimental conditions. The present study is the first time that the loss of responsiveness to CORT by pituitary cells during late chick embryonic development has been demonstrated using the same experimental and assay conditions also used to show stimulatory effects on e12. Growth hormone secretion by the pituitary is at a maximum just prior to hatching [29], which may be why CORT is unable to elicit a response over already increased GH secretion. It is possible that the rate of GH secretion is increasing during the final third of embryonic development (e14–e20) in response to the endogenous increase in serum CORT, and therefore, is unresponsive to glucocorticoids. Alternatively, the change in responsiveness may reflect differences in receptor levels, occupancy, or involvement.

Growth hormone-releasing hormone is 1 of the 2 hypothalamic peptides that tightly regulate GH secretion from the pituitary. In the present study, GHRH was used to stimulate the release of stored GH. Growth hormone responses to CORT were seen only following release of stored GH by inclusion of GHRH. At e12, there are low levels of GH protein and, most likely, the rate of synthesis is low as well, therefore, addition of GHRH will not increase the rate of GH release. At e20, as there are high levels of GH being synthesized and secreted already, GHRH treatment may not produce a significant difference in the amount of GH released.

Our results demonstrate that the somatotroph response to CORT is modulated by T_3 only on specific embryonic ages in development. A functional thyroid and thyroid-stimulating hormone (TSH) is necessary for normal somatotroph growth and GH secretion. Defects affecting the thyroid or the thyrotrophs of the pituitary markedly affect GH secretion. In α-GSU−/− mice, the number of somatotrophs is severely decreased in the pituitary [30]. Likewise, in Pax8−/− mice, an athyroid mutant mouse, the number of somatotrophs is greatly reduced, as measured in 21-day-old mice [31]. In this report, we demonstrated that e12 pituitary cells are unresponsive to the combination of CORT and T_3 after 3 days in culture; however, on e14 and e16, T_3 suppresses the CORT-induced increase in GH secretion. On e18, T_3 alone increases GH secretion over basal levels, and this effect is lost by e20. Regulation of GH by T_3 is complex and most likely due to a multitude of convergent pathways. In the rat pituitary cell line GH_3, T_3 dramatically increases GH transcription independently from glucocorticoid treatment [26], suggesting that the mechanisms of action by which CORT and T_3 induce GH are separate. Pituitary GH was markedly increased in fetal rats from T_3-treated dams compared to fetuses of control dams [32]. In our study, there was no combined effect of CORT and T_3 on e18 or e20 relative to basal and CORT-treated chicks. A study performed in the rainbow trout demonstrated that combined treatment of DEX and T_3 significantly increased GH, and GHRH also modulated this response [28]. The thyroid anlagen is detectable as early as e2 in the chicken [33], and detectable levels of circulating T_3 can be detected by e5 [34]. Thyroid hormone is also modulated by 3 deiodinases, D1, D2, and D3, all of which have different specificities for the 3 different forms of thyroid hormone. In the embryonic chicken, D2 is predominant in the brain [34]. Brain deiodinase activity is biphasic, where it is first detectable at e5, but virtually disappears between e14 and e17 in the chicken, with the highest concentrations occurring on e13 and e20, suggesting that T_4 cannot be quickly converted to T_3 or T_3 cannot be quickly inactivated [34]. Other studies have demonstrated that extended treatment with CORT and T_3 is highly stimulatory on e11 [11], but acute treatment had no affect on GH secretion on e20 [10]. This finding was also shown in e20 fetal and neonatal day 2 rat pituitary cell cultures [35]. Although the concentration (0.25 mM) was 1000-fold higher than our treatment, T_3 had no effect on e20 cells, but was stimulatory after birth [35]. Again, in cultured human fetal pituitary cells, T_3 decreased basal GH secretion and attenuated the GHRH-induced increase in GH secretion [36]. Addition of DEX restored GHRH-responsiveness of the cells [36]. One explanation for the differing results may be that these studies used different culture conditions and different assays to determine the effects of glucocorticoids and T_3 on GH secretion. However, it is well known that glucocorticoids and thyroid hormones interact to affect GH gene transcription in rats [37–40]. Thyroid hormones induce GH synthesis 5 to 20-fold, and cortisol increases this response 2 to 6-fold higher in the rat pituitary GH_3 cell line [41]. Both alone and in combination, T_3 and DEX increased GH gene transcription, and this effect was still observed while thyroid hormone (TR) and GR mRNA levels were reduced [41]. This is evidence that receptor presence is not the only factor regulating GH gene transcription. There could also be a confounding interaction between CORT and T_3, since in e18 chicken embryos, glucocorticoids sig-

nificantly increase T_3 serum levels while concomitantly decreasing T_4 and rT_3 [25]. Thyroid hormone is also part of the nuclear/steroid receptor superfamily and binds to promoters to affect gene transcription. Studies have shown that TR can form preinitiation complexes (PIC) by recruiting TFIIB or activate an already existing one to start transcription [42,43]. Thus, it is possible that formation or activation of PIC by TR and GR/MR is one modulator of GH gene transcription. Whether the responses involve direct interactions of nuclear receptors or not, the present results demonstrate that the nature of the GH response to CORT and T_3 changes during development, where T_3 is stimulatory around the time of GH differentiation and is inhibitory or ineffectual during the last half of chick embryonic development.

Treatment with a GR-specific antagonist, ZK98, alone abolished the CORT-mediated induction of GH secretion by e12 pituitary cells, and treatment with both antagonists in combination also blocked CORT-stimulated GH secretion. This finding suggests that CORT actions on GH are mediated primarily through GR. Induction of GH by CORT via both MR and GR was demonstrated previously by estimating the number of GH-containing cells using ICC when cells were treated with these same receptor-specific antagonists [12]. Corticosterone-induced GH cell differentiation in cultured e12 pituitary cells was blocked only when treated with both the MR-specific antagonist, SPIRO, and the GR-specific antagonist, ZK98. Glucocorticoid receptor was expressed in all pituitary cells at e12, and MR was co-localized with GH greater than 90% of the time in e12 somatotrophs, as shown by ICC and dual-label immunofluorescence, respectively [12]. Glucocorticoid receptor and GH were co-localized in 98% of cells in the rat anterior pituitary as well [44]. Differences in the involvement of MR between our previous report [12] and our current study may be due to the assays used. Again, ICC measures the presence of GH in a cell, whereas RIA measures the amount of GH secreted. It is possible that secretion of GH levels detectable by RIA may require a higher amount of CORT, whereas production of GH and packaging into secretory granules detectable by ICC can be induced with lower levels of CORT. It is believed that in the adult brain, MRs are occupied with basal levels of CORT most of the time and that GRs are mostly unoccupied [45]. Further, when the circulating concentration of CORT increases, GRs are activated after MR saturation in neuronal tissue [46]. Thus, GH production in response to low levels of CORT may be mediated primarily through MR, but when CORT concentrations rise, GRs become activated as well and GH secretion is activated. This finding is further supported by the finding that GR and MR are known to heterodimerize, and upon heterodimerization, DNA-specific binding is greatly increased [47]. Because the 2 receptors are expressed in the majority of pituitary somatotrophs on e12, heterodimerization of receptors may play an important role in the responsiveness of GH to CORT at this age.

In conclusion, glucocorticoids increase GH secretion between e12 and e18 of chick embryonic development until just prior to hatching, when the cells are no longer responsive to CORT. Growth hormone releasing hormone and T_3 modulate this effect. Glucocorticoid receptor primarily mediates this response at the midpoint of development. However, the downstream targets of GR and the mechanism by which it induces GH gene expression are still unclear.

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References

[1] Ohlsson C, Bengtsson Ba, Isakson OG, Andreassen TT, Slootweg MC. Growth hormone and bone. Endocr Rev 1998;19:55–79.
[2] Copeland KC, Nair KS. Acute growth hormone effects on amino acid and lipid metabolism. J Clin Endocrinol Metab 1994;78:1040–7.
[3] Dorshkind K, Horseman ND. The roles of prolactin, growth hormone, insulin-like growth factor-I, and thyroid hormones in lymphocyte development and function: insights from genetic models of hormone and hormone receptor deficiency. Endocr Rev 2000;21:292–312.
[4] Vasilatos-Younken R, Cravener TL, Cogburn LA, Mast MG, Wellenreiter RH. Effect of pattern of administration on the response to exogenous, pituitary-derived chicken growth hormone by broiler-strain pullets. Gen Comp Endocrinol 1988;71:268–83.
[5] Porter TE, Couger GS, Morpurgo B. Evidence that somatotroph differentiation during chicken embryonic development is stimulated by a blood-borne signal. Endocrinology 1995;136:3721–8.
[6] Morpurgo B, Dean CE, Porter TE. Identification of the blood-borne somatotroph-differentiating factor during chicken embryonic development. Endocrinology 1997;138:4530–5.

[7] Jenkins SA, Muchow M, McMurtry JP, Porter TE. Administration of adrenocorticotrophic hormone during chicken embryonic development prematurely induces pituitary growth hormone cells. Endocrinology 2007;148:3914–21.
[8] Dean CE, Morpurgo B, Porter TE. Induction of somatotroph differentiation in vivo by corticosterone administration during chicken embryonic development. Endocrine 1999;11:151–6.
[9] Dean CE, Porter TE. Regulation of somatotroph differentiation and growth hormone (GH) secretion by corticosterone and GH-releasing hormone during embryonic development. Endocrinology 1999;140:1104–10.
[10] Porter TE, Dean KJ. Regulation of chicken embryonic growth hormone secretion by corticosterone and triiodothyronine: evidence for a negative synergistic response. Endocrine 2001;14:363–8.
[11] Liu L, Dean CE, Porter TE. Thyroid hormones interact with glucocorticoids to affect somatotroph abundance in chicken embryonic pituitary cells in vitro. Endocrinology 2003;144:3836–41.
[12] Bossis I, Nishimura S, Muchow M, Porter TE. Pituitary expression of type I and type II glucocorticoid receptors during chicken embryonic development and their involvement in growth hormone cell differentiation. Endocrinology 2004;145:3523–31.
[13] Funder JW. Glucocorticoid and mineralocorticoid receptors: biology and clinical relevance. Ann Rev Med 1997;48:231–40.
[14] Raynaud JP, Bouton MM, Moguilewsky M, et al. Steroid hormone receptors and pharmacology. J Steroid Biochem 1980;12:143–57.
[15] Reul JM, de Kloet ER, van Sluijs FJ, Rijnberk A, Rothuizen J. Binding characteristics of mineralocorticoid and glucocorticoid receptors in dog brain and pituitary. Endocrinology 1990;127:907–15.
[16] Porter TE, Ghavam S, Muchow M, Bossis I, Ellestad L. Cloning of partial cDNAs for the chicken glucocorticoid and mineralocorticoid receptors and characterization of mRNA levels in the anterior pituitary gland during chick embryonic development. Domest Anim Endocrinol 2007;33:226–39.
[17] Porter TE, Cougar GS, Dean CE, Hargis BM. Ontogeny of growth hormone (GH)-secreting cells during chicken embryonic development: initial somatotrophs are responsive to GH-releasing hormone. Endocrinology 1995;136:1850–6.
[18] Houston B, Peddie D, Goddard C. Monoclonal antibody based sandwich enzyme-linked immunosorbent assay for chicken growth hormone. Br Poult Sci 1991;32:633–44.
[19] Krishnan KA, Proudman JA, Bahr JM. Purification and partial characterization of isoforms of luteinizing hormone from the chicken pituitary gland. Comp Biochem Physiol Biochem Mol Biol 1994;1082:253–64.
[20] Ellestad LE, Carre W, Muchow M, et al. Gene expression profiling during cellular differentiation in the embryonic pituitary gland using cDNA microarrays. Physiol Genomics 2006;25:414–25.
[21] Arriza JL, Simerly RB, Swanson LW, Evans RM. The neuronal mineralocorticoid receptor as a mediator of glucocorticoid response. Neuron 1988;1:887–900.
[22] Arizza JL, Weinberger C, Cerelli G, et al. Cloning of human mineralocorticoid receptor complementary DNA: structural and functional relationship with the glucocorticoid receptor. Science 1987;237:268–75.
[23] Rupprecht R, Reul JM, van Steensel B, et al. Pharmacological and functional characterization of human mineralocorticoid and glucocorticoid receptor ligands. Eur J Pharmacol: Mol Pharmacol Sec 1993;247:145–54.
[24] Bossis I, Porter TE. Ontogeny of corticosterone-inducible growth hormone-secreting cells during chick embryonic development. Endocrinology 2000;141:2683–90.
[25] Darras VM, Kotanen SP, Geris KL, Berghman LR, Kuhn ER. Plasma thyroid hormone levels and iodothyronine deiodinases activity following an acute glucocorticoid challenge in embryonic compared with posthatch chickens. Gen Comp Endocrinol 1996;104:203–12.
[26] Evans RM, Birnberg NC, Rosenfeld MG. Glucocorticoid and thyroid hormones transcriptionally regulate growth hormone gene expression. Proc Natl Acad Sci USA 1982;79:7659–63.
[27] Rodriguez-Garcia M, Join T, Santose A, Perez-Castillo A. Effect of perinatal hypothyroidism on the developmental regulation of rat pituitary growth hormone and thyrotropin genes. Endocrinology 1995;136:4339–50.
[28] Luo D, McKeown BA. The effect of thyroid hormone and glucocorticoids on carp growth hormone-releasing factor (GRF)-induced growth hormone (GH) release in rainbow trout (*Oncorhynchus mykiss*). Comp Biochem Physiol A 1991;99:621–6.
[29] Scanes CG. The physiology of growth, growth hormone, and other growth factors in poultry. CRC Crit Rev Poult Biol 1987;1:51–105.
[30] Stahl JH, Kendall SK, Brinkmeier ML, Greco TL, Watkins-Chow DE, Campos-Barros A, et al. Thyroid hormone is essential for pituitary somatotropes and lactotropes. Endocrinology 1999;140:1884–92.
[31] Friedrichsen S, Christ S, Heuer H, et al. Expression of pituitary hormones in the Pax8−/− mouse model of congenital hypothyroidism. Endocrinology 2004;145:1276–83.
[32] De Escobar GM, Calvo R, Del Rey FE, Obregon MJ. Differential effects of thyroid hormones on growth and thyrotropic hormones in rat fetuses near term. Endocrinology 1993;132:2056–64.
[33] McNabb FMA. Thyroid function in embryonic and early posthatch chickens and quail. Poult Sci 1989;68:990–8.
[34] Valverde-R C, Aceves C, Reyes-Z E. Ontogenesis of iodothyronine deiodinases activities in brain and liver of the chick embryo. Endocrinology 1993;132:867–72.
[35] Ezzat S, Laks D, Oster J, Melmed S. Growth hormone regulation in primary fetal and neonatal rat pituitary cell cultures: the role of thyroid hormone. Endocrinology 1991;128:937–43.
[36] Mulchahey JJ, Di Blasio AM, Jaffe RB. Effects of growth hormone (GH)-releasing hormone and somatostatin on GH secretion from individual human and monkey fetal anterior pituitary cells: modulation by thyroid hormones and glucocorticoids. J Clin Endocrinol Metab 1988;66:395–401.
[37] Shapiro LE, Samuels HH, Yaffe BM. Thyroid and glucocorticoid hormones synergistically control growth hormone mRNA in cultured GH1 cells. Proc Natl Acad Sci USA 1978;75:45–9.
[38] Nogami H, Yokose T, Tachibana T. Regulation of growth hormone expression in fetal rat pituitary gland by thyroid or glucocorticoid hormone. Am J Physiol 1995;268:E262–7.
[39] Nyborg JK, Nguyen AP, Spindler SR. Relationship between thyroid and glucocorticoid receptor occupancy, growth hormone gene transcription and mRNA accumulation. J Biol Chem 1984;259:12377–81.
[40] Yaffe BM, Samuels HH. Hormonal regulation of the growth hormone gene. Relationship of the rate of transcription to the level of nuclear thyroid hormone–receptor complexes. J Biol Chem 1984;259:6284–91.
[41] Williams GR, Franklyn JA, Sheppard MC. Thyroid hormone and glucocorticoid regulation of receptor and target gene

mRNAs in pituitary GH3 cells. Mol Cell Endocrinol 1991;80: 127–38.

[42] Kim MK, Lee JS, Chung JH. In vivo transcription factor recruitment during thyroid hormone receptor-mediated activation. Proc Natl Acad Sci USA 1999;96:10092–7.

[43] Baniahmad A, Ha I, Reinberg D, Tsai S, Tsai MJ, O'Malley BW. Interaction of human thyroid hormone receptor beta with transcription factor TFIIB may mediate target gene derepression and activation by thyroid hormone. Proc Natl Acad Sci USA 1993;90:8832–6.

[44] Ozawa H, Ito T, Ochiai I, Kawata M. Cellular localization and distribution of glucocorticoid receptor immunoreactivity and the expression of glucocorticoid receptor messenger RNA in rat pituitary gland. Cell Tissue Res 1999;295:207–14.

[45] De Kloet RE, Oitzl MS, Joels M. Functional implication of brain corticosteroid receptor diversity. Cell Mol Neurobiol 1993;13:433–55.

[46] McEwan BS, Angulo J, Cameron H, et al. Paradoxical effects of adrenal steroids on the brain: protection versus degeneration. Biol Psychiatry 1992;31:177–99.

[47] Trapp T, Rupprecht R, Castrén M, Reul JM, Holsboer F. Heterodimerization between mineralocorticoid and glucocorticoid receptor: a new principle of glucocorticoid action in the CNS. Neuron 1994;13:1457–62.